Variable lymphocyte receptors in hagfish

Zeev Pancer*, Nil Ratan Saha†, Jun Kasamatsu‡§, Takashi Suzuki‡, Chris T. Amemiya†, Masanori Kasahara‡¶, and Max D. Cooper*‖**††‡‡

Division of Developmental and Clinical Immunology, Departments of *Medicine, ‖Pediatrics, and **Microbiology, and ††Howard Hughes Medical Institute, University of Alabama, Birmingham, AL 35294; †Molecular Genetics Program, Benaroya Research Institute at Virginia Mason, Seattle, WA 98101; ‡Department of Biosystems Science, School of Advanced Sciences, Graduate University for Advanced Studies (Sokendai), Hayama, Kanagawa 240-0193, Japan; §Department of Biology, Graduate School of Engineering and Science, Yamagata University, Yamagata 990-8567, Japan; and ¶Department of Pathology, Hokkaido University Graduate School of Medicine, Sapporo, Hokkaido 060-8638, Japan

A previously uncharacterized type of variable lymphocyte receptors (VLR) was identified recently in the Sea lamprey. This jawless vertebrate generates an extensive VLR repertoire through differential insertion of neighboring diverse leucine-rich repeat (LRR) cassettes into an incomplete germ-line *VLR* gene. We report here VLR homologs from two additional lamprey species and the presence of two types of *VLR* genes in hagfish, the only other order of contemporary jawless vertebrates. As in the Sea lamprey, the incomplete hagfish germ-line *VLR-A* and *-B* genes are modified in lymphocyte-like cells to generate highly diverse repertoires of VLR-A and -B proteins via a presently undetermined mechanism. This jawless-fish mode of VLR diversification starkly contrasts with the rearrangement of Ig V(D)J gene segments used by all jawed vertebrates to produce diverse repertoires of T and B lymphocyte antigen receptors. The development of two very different strategies for receptor diversification at the dawn of vertebrate evolution ≈500 million years ago attests to the fitness value of a lymphocyte-based system of anticipatory immunity.

adaptive immunity | agnatha | jawless fish

Hagfish are grouped with the lamprey in the cyclostome taxon, representing a phylogenetically ancient lineage that diverged before the jawed vertebrates (1–3). Because of their unique phylogenetic position, these eel-shaped jawless fish have been studied extensively in search for the origin of vertebrate adaptive immunity. Like lamprey, the hagfish have heterogeneous blood leukocyte populations, including cells morphologically similar to mammalian lymphocytes (4–9). Both hagfish and lamprey have been reported to produce specific agglutinins and opsonins in response to particulate antigens (9–12) and to undergo accelerated rejection of second-set skin allografts (10, 13). However, neither agnathan has been shown to possess the essential components that gnathostomes use for adaptive immunity, namely Ig, T cell receptors, recombination activating genes RAG1 and -2 for V(D)J rearrangement, and MHC class I and II molecules (14–18).

Recently, we identified a previously undescribed type of variable lymphocyte receptors (VLR) in the Sea lamprey (19). VLR are proteins consisting of leucine-rich repeats (LRR) that are assembled into functional receptors through somatic diversification of the single incomplete germ-line *VLR* gene (*gVLR*) in lamprey lymphocytes. Here we describe VLR homologs from species representing two other lamprey families and from two hagfish species, representing the remaining cyclostome order.

Materials and Methods

Animals. Live specimens of Pacific hagfish *Eptatretus stoutii* (30–60 cm long) were purchased from Marinus (Long Beach, CA) and maintained for 2 months at 12°C in artificial sea water (Oceanic Systems, Dallas). Larvae (15–20 cm long) of the American brook lamprey (*Lampetra appendix*) and Northern brook lamprey (*Ichthyomyzon fossor*) were from tributaries to the Great Lakes (Lamprey Services, Ludington, MI).

Hagfish were sedated by immersion for 15 min in 0.5 g/liter

MS222 (Sigma) buffered to pH 7 before i.p. injection with an antigen/mitogen mixture in 0.5 ml of hagfish PBS (per liter, 28 g of NaCl/0.2 g of KCL/1.44 g of Na_2HPO_4/0.24 g of KH_2PO_4, pH 7.4, 1 osmol). The mixture contained 10^9 live *Escherichia coli* TG1 bacteria, 10^9 sheep erythrocytes (Colorado Serum, Denver) and 100 μg each of phytohemagglutinin and pokeweed mitogen (Sigma). Immune stimulation was repeated at weekly intervals and, 4 days after the fourth stimulation, blood was collected with a syringe from the tail blood sinus and diluted 1:1 with hagfish PBS containing 30 mM EDTA. Buffy coat leukocytes collected after 5-min centrifugation at 50 × *g* were sorted by their light-scatter characteristics as described (8, 9) by using a MoFlo cytometer (Cytomation, Ft. Collins, CO).

Hagfish VLR. Inshore hagfish *Eptatretus burgeri* VLR homologs were identified by using lamprey VLR as BLAST queries against the database of expressed sequence tags from leukocyte RNA of unstimulated animals nos. 7 and 8 (16). Clones with significant matches were sequenced on both strands, 64 VLR-A and 15 VLR-B cDNA clones. For the Pacific hagfish, unseparated blood cells and buffy coat leukocytes from three unstimulated individuals (nos. 1–3 and 6) and buffy coat leukocytes from two immunostimulated animals (nos. 4 and 5) were used for extraction of blood genomic DNA and leukocyte RNA. Extraction of RNA was with TRIzol Reagent (Invitrogen), and PolyA RNA was selected with the Dynabeads mRNA purification Kit (Dynal, Lake Success, NY). First-strand cDNA synthesis was primed with 20 pmol of HgVLRA.F1 (Table 2, which is published as supporting information on the PNAS web site) for VLR-A or HgVLRB.F1 for VLR-B, using the SuperScript III First-Strand cDNA Synthesis kit (Invitrogen), and the products were column-purified (QIAquick PCR purification, Qiagen, Valencia, CA). VLRs were then PCR-amplified by using Expand High Fidelity PCR (Roche Applied Science, Indianapolis) from the cDNA or from genomic DNA, in 50-μl reactions containing 1 μl each of the sets of forward and reverse primers (F1 or F2 and R1 or R2) at 10 pmol/μl, 5 μl of 10× buffer, 36.25 μl of double distilled water, 5 μl of cDNA or genomic DNA (250 ng), and 0.75 μl of the polymerase. Reactions were amplified by using one cycle of 94°C, 1 min; then 35 cycles of 94°C, 30 sec; 58°C, 30 sec; 72°C, 1 min; and a final 7-min elongation at 72°C. Products were column-purified, cloned in pCRII-TOPO (Invitrogen), and the inserts were sequenced. For the Pacific hagfish, 109 VLR-A RT-PCR clones were sequenced (four contained in-frame stop codons; not shown), and 36 genomic mature *VLR-A* amplicons (two contained in-frame stop codons). For VLR-B, 37 RT-PCR
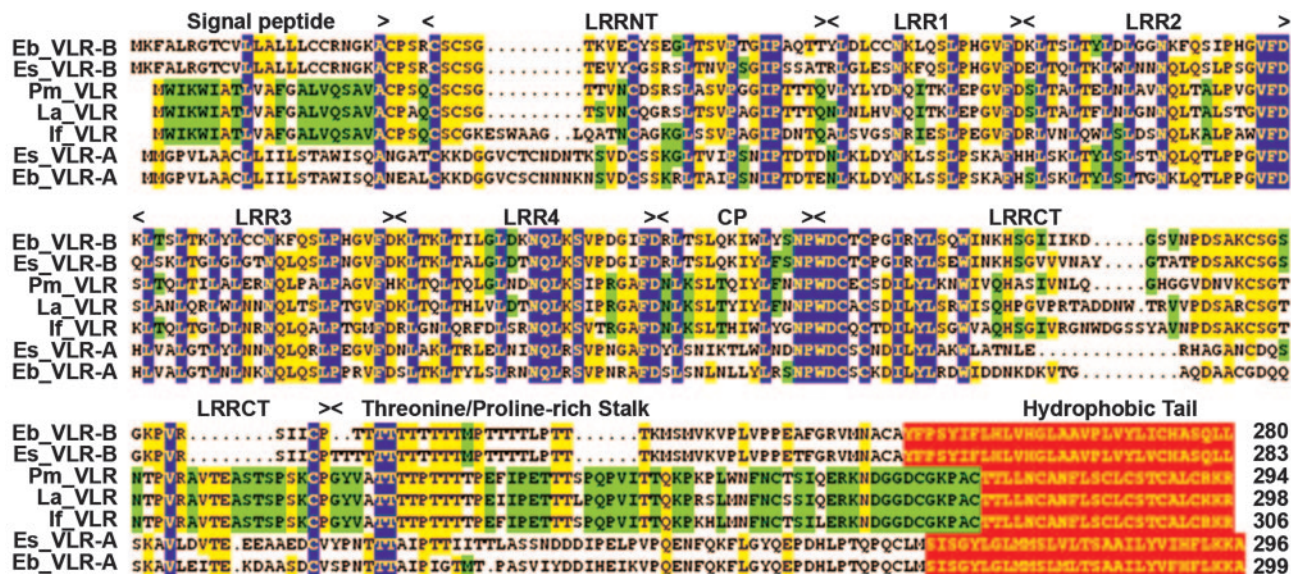

Fig. 1. Evolutionarily conserved agnathan VLRs. VLR amino acid sequences representing the Inshore hagfish (*E. burgeri*), Pacific hagfish (*E. stoutii*), Sea lamprey (*P. marinus*; GenBank accession no. AY577946), American brook lamprey (*L. appendix*), and Northern brook lamprey (*I. fossor*). Blue shade, 100% identity; yellow, 60–99%; green, 40–59%; and red, hydrophobic tail region.

clones were sequenced (one contained an in-frame stop codon) and 38 genomic mature *VLR-B* amplicons (four contained in-frame stop codons; not shown). Liver genomic DNA from Inshore hagfish no. 9 (16) was used for PCR cloning and sequencing mature *VLR*s, four mature *VLR-A* amplicons (two contained in-frame stop codons), and three mature *VLR-B* amplicons.

Nonparasitic Lamprey VLR. First-strand cDNA was synthesized as above by using the reverse primer VLR_3UT.R (Sea lamprey 3′ UTR primer, Table 2). For the American brook lamprey, the forward primer was Slit.F (Sea lamprey 5′ UTR primer) and for the Northern brook lamprey, LRR_N.F1 (another Sea lamprey 5′ UTR primer). In total, 13 unique VLR clones of the American brook lamprey and seven of the Northern brook lamprey were sequenced.

Bacterial Artificial Chromosome (BAC) Libraries and Clones. An Inshore hagfish BAC library (20) was screened by PCR by using VLR primers as above (F1 or F2 and R1 or R2). The Pacific hagfish BAC library (VMRC23) was constructed from EcoRI partial digests of erythrocyte DNA from a single specimen in the vector pCCBACE1 (Epicentre Technologies, Madison, WI). This library consists of ≈184,000 recombinants and encompasses ≈5× coverage of the hagfish genome. The entire library was screened by hybridization with 5′ and 3′ VLR-A and -B probes, and positive clones were authenticated by PCR. One BAC for each VLR type from the Pacific and Inshore hagfish was sequenced at ≈10× coverage and assembled into contigs (Macrogen, Seoul, Korea). In case of incomplete sequence of the inserts, only portions containing the gVLR and LRR cassettes were included with uncaptured gaps in the contigs: Eb_gVLR-A, 43,362 bp; Eb_gVLR-B, 92,072 bp; Es_gVLR-A, 81,648 bp; and Es_gVLR-B, 76,730 bp.
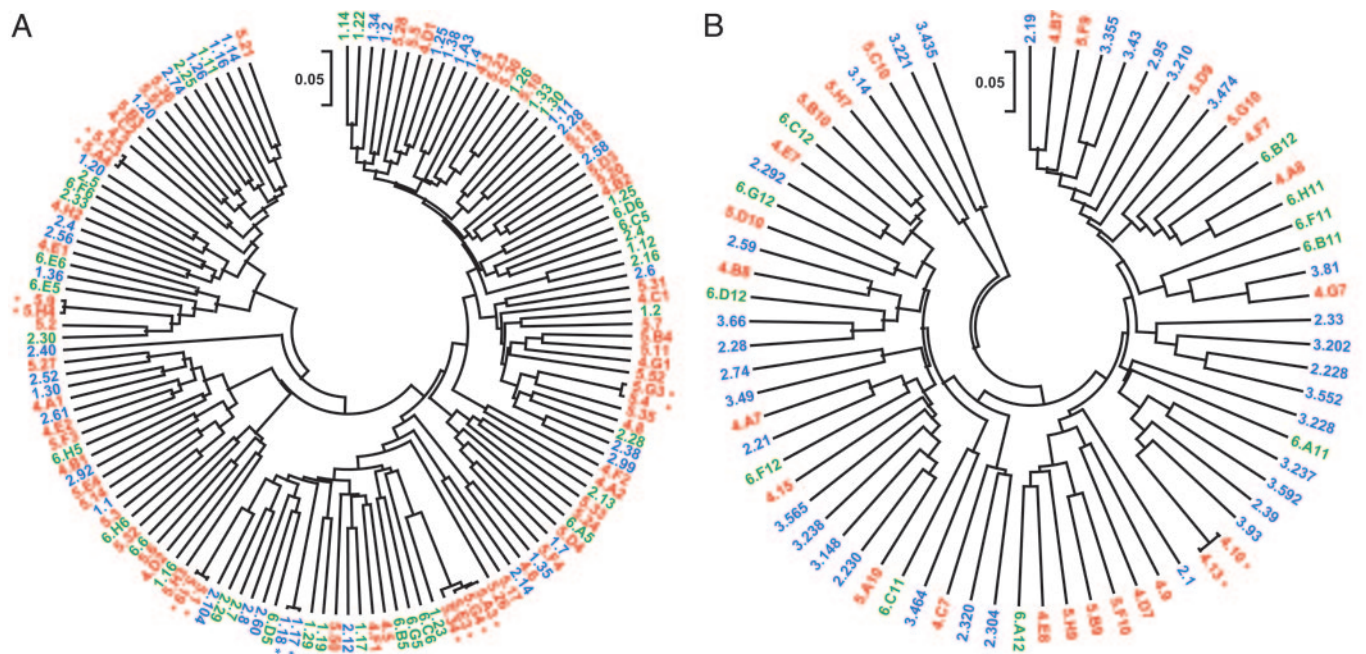
Sequence Analysis. Neighbor-joining and Unweighted Pair Group Method with Arithmatic Mean (UPGMA) trees were constructed with the pairwise deletion option by using MEGA3 molecular evolutionary genetics analysis software (21). Prediction of genes in the BAC inserts was accomplished by using local BLAST downloaded from ftp://ftp.ncbi.nlm.nih.gov/blast/ executables and the GenScan server, genes.mit.edu/GENSCAN. html.

Results and Discussion

Cyclostome VLR Homologs. Two distinct types of VLR, VLR-A and -B, were identified among expressed sequence tags from 12,000 leukocyte cDNA clones of the Inshore hagfish, *E. burgeri* (16). Matching VLR were then cloned by RT-PCR from transcripts of lymphocyte-like cells of the Pacific hagfish, *E. stoutii*. Fig. 1 depicts an alignment of the amino acid sequences of hagfish VLR-A and -B, the Sea lamprey VLR (*Petromyzon marinus*) and VLRs of two nonparasitic lampreys, American brook lamprey (*L. appendix*) and Northern brook lamprey (*I. fossor*). These VLRs share similar structural domains: a signal peptide, N-terminal LRR (LRRNT); 18-residue LRR1 followed by a variable number of 24-residue LRRs; a 13-residue connecting peptide (CP); and C-terminal LRR (LRRCT). At the beginning of the C terminus, the lamprey VLR and hagfish VLR-B have a threonine/proline-rich region, but this region is not well conserved in the hagfish VLR-A. All VLR proteins end with a hydrophobic tail region that is required for modification of the protein to add a glycosyl-phosphatidyl-inositol (GPI) cell surface membrane anchor. Like the sea lamprey VLR (19), hagfish VLR-A was predicted to be a GPI-anchored protein, although no ω cleavage site was identified (DGPI, http://129.194.185. 165/dgpi); the C-terminal hydrophobicity profile for VLR-B is also predictive of GPI modification.

Transcripts of hagfish VLR are abundant in lymphocyte-like cells but not in myeloid cells or erythrocytes sorted by their light-scatter characteristics. VLR-A transcript levels were ≈3-fold higher than VLR-B levels in blood leukocyte samples (not shown). Both VLR types of the Pacific hagfish are highly heterogeneous (Fig. 2), exhibiting variable numbers of the 24-residue LRR modules and pronounced LRR sequence diversity. Comparable diversity was observed for VLR-A (*n* = 66) and -B (*n* = 18) sequences from Inshore hagfish (Fig. 5, which is published as supporting information on the PNAS web site). Interestingly, five clusters of two to four VLR-A clones that were identical or differed by only one to two residues were found among the 40 transcripts from hagfish no. 5 (marked by asterisks in Fig. 2*A*), which was given four weekly injections of an antigen
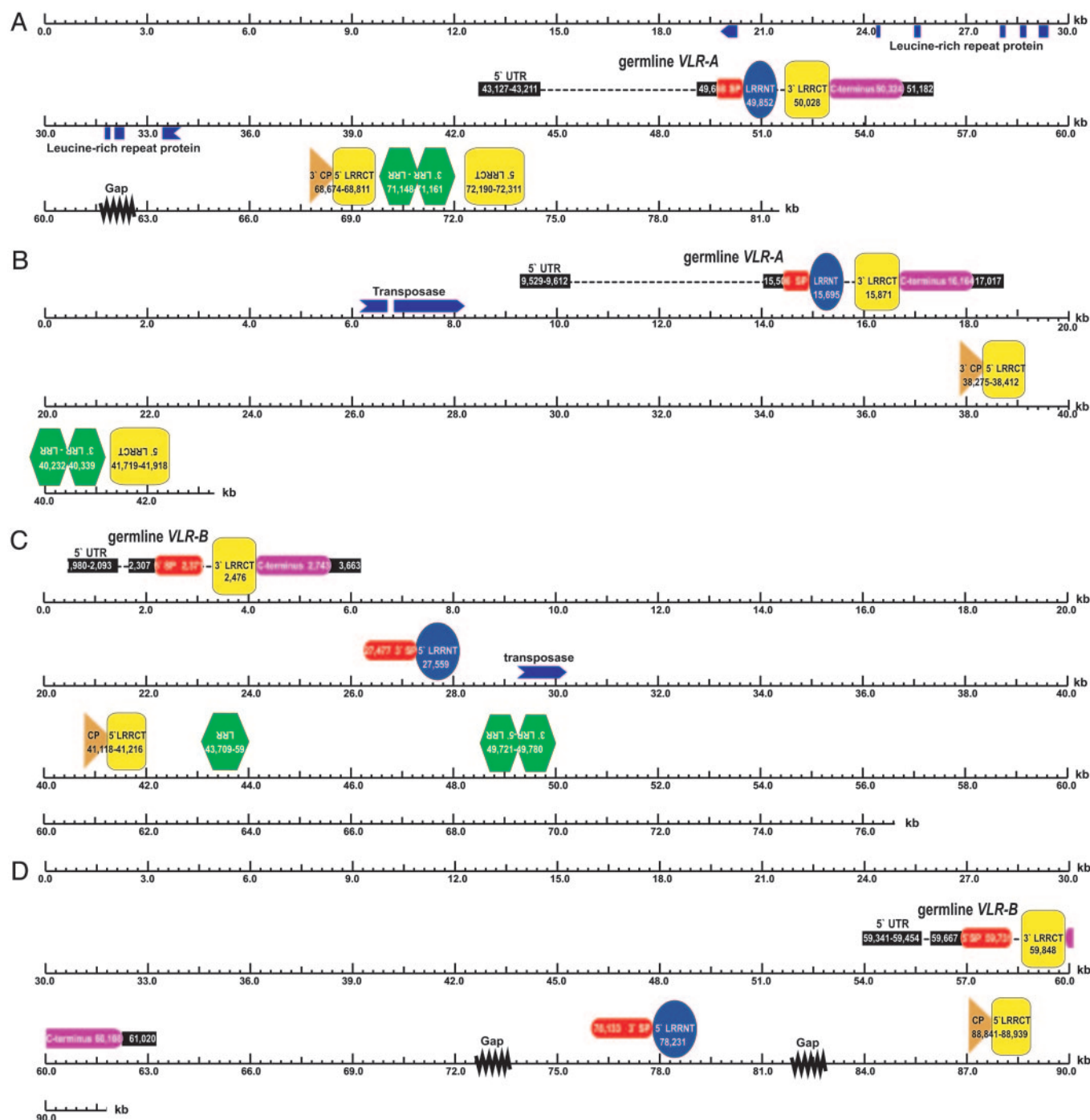
Fig. 2. Genetic distance among Pacific hagfish VLR diversity regions (LRRNT to LRRCT). Proteins predicted from PCR-amplified lymphocyte-like cDNA clones or blood genomic PCR amplicons from five animals. Bars represent 5% amino acid divergence. (*A*) VLR-A (*n* = 139); (*B*) VLR-B (*n* = 70). Green, unstimulated; red, immunostimulated; and blue, genomic mature *VLR*; asterisk-related sequences.

and mitogen mixture. The finding that 30% of the VLR-A transcripts from this hagfish consisted of clusters of related sequences may indicate clonal expansion of VLR-A-bearing lymphocytes. The clones with one to two amino acid substitutions could reflect additional VLR diversification through somatic hypermutation. The elucidation of the implied role for clonal expansion and diversification in hagfish immunity will require additional experiments.

The dataset of unique sequence Pacific hagfish VLR-A (*n* = 130) reveals two to six copies per transcript of the 24-residue LRRs (*n* = 527; average, four). In the VLR-B dataset (*n* = 69), there are one to six copies of the 24-residue LRRs (*n* = 195; average, 2.8), whereas in the set of 129 Sea lamprey VLR (ref. 19; GenBank accession nos. AY577943–AY578059), there were one to nine copies of 24-residue LRRs (*n* = 325; average, 2.5). We then analyzed separately in a neighbor-joining phylogenetic tree the individual components of these VLR, except for LRRNT and LRRCT that were too diverse among the species for reliable alignment (Table 1; 328 LRR1 domains, 328 CP domains, and 1,047 single domains of the 24-residue LRRs). The clusters were nearly exclusively of the same type and species origin, i.e., Pacific hagfish VLR-A, VLR-B, or Sea lamprey VLR clustering. There were no instances of identical LRR domains between the different VLR types. However, a large portion of the LRR1 and CP domains within hagfish VLR-A and lamprey VLR clusters were identical (Table 1). In contrast, the LRR1

domains in hagfish VLR-B were 98% unique; the sets of 24-residue LRRs also consisted predominantly of unique sequences: 97% were unique in hagfish VLR-B, 90% in VLR-A, and 83% in the Sea lamprey VLR. This remarkably high degree of diversity is especially remarkable, given that consensus sequences derived for each of the 24-residue LRR types share at least 10 framework residues.

Hagfish *VLR* Genes. Genomic organization of the Pacific and Inshore hagfish *VLR* loci was determined from sequences of large insert genomic clones isolated from BAC libraries, one BAC for each VLR type (Fig. 3). Only one copy of each of the g*VLRs* was identified in hagfish genomes (not shown). The sequences and organization of the loci are nearly identical in both species and fairly conserved between g*VLR-A* and *-B*. Hagfish g*VLR* begin with a 5′ UTR followed by two coding regions (Fig. 4*A*). As in the Sea lamprey g*VLR*, the 5′ UTR is split by an intron, 6.4 kb long in the Pacific hagfish g*VLR-A* and 220 bp long in g*VLR-B*. The first coding region in the hagfish g*VLR* encodes the signal peptide and an LRRNT domain in g*VLR-A* and only residues 1–13 of the 23-residue signal peptide in g*VLR-B*. Next, there are short intervening sequences of 171 and 105 bp for g*VLR-A* and *-B*, respectively. The second coding region consists of the 3′ end of LRRCT and the C terminus, as in the Sea lamprey g*VLR*, except that the lamprey region coding for the 5′ end of LRRCT is missing. The hagfish g*VLR* are

Table 1. Components of unique hagfish and Sea lamprey VLR

	Unique LRR motifs			
	LRR1 (18 aa)	CP (13 aa)	Diversity LRR (24 aa)	Diversity LRR consensus*
Es_VLR-A	77/130 (59%)	71/130 (55%)	477/527 (90%)	–L––L––L–L––NqL––1P–G–FD
Es_VLR-B	68/69 (98%)	46/69 (67%)	190/195 (97%)	KLT–Lt–L–L––NqL–S–P–GvFD
Pm_VLR	68/129 (53%)	36/129 (28%)	269/325 (83%)	–L––L––L–L––NQL–––P–G–FD

*Consensus: capital letters, 80–100% identity; small letters, 60–79%.

Fig. 3. Hagfish *VLR* gene loci. (*A*) Pacific hagfish *VLR-A*. (*B*) Inshore hagfish *VLR-A*. (*C*) Pacific hagfish *VLR-B*. (*D*) Inshore hagfish *VLR-B*. Sequence of inserts from four BAC clones, with uncaptured gaps marked. Location of *VLR* germ-line genes and flanking cassettes, in reverse (indicated by inverted orientation) or forward orientation, is indicated in kilobases (graphics are out of scale). GenScan gene predictions are indicated in blue, an unrelated *LRR* gene upstream from the Pacific hagfish germ-line *VLR-A* gene and two flanking transposase ORFs in the Inshore hagfish *VLR-A* and Pacific hagfish *VLR-B* loci.

compact, 671 bp from start-to-stop codons in *gVLR-A* and 410 bp in *gVLR-B*.

The hagfish *gVLR* loci harbor cassettes encoding diverse LRR motifs located ≈20–40 kb downstream from the germ-line genes (Fig. 3). In the *VLR-A* locus, there is a cassette encoding six to eight terminal residues of a diverse CP domain and a 5′ LRRCT that includes a four-residue identical overlap with the *gVLR-A* 3′ LRRCT. Farther downstream, there is a cassette of two diverse LRRs positioned in reverse orientation

relative to the *gVLR-A* and then an inverted incomplete 5′ LRRCT. In the *gVLR-B* locus, there is a cassette encoding residues 7–23 of the signal peptide and a 5′ LRRNT, then a diverse CP domain and 5′ LRRCT, one inverted LRR and, farther downstream, another inverted LRR cassette consisting of the 12-terminal and 8-proximal residues of LRRs. No other diverse LRR modules were identified in flanking DNA spanning ≈50 kb upstream and ≈70 kb downstream from the *gVLR*s. However, diverse LRR elements likely exist elsewhere
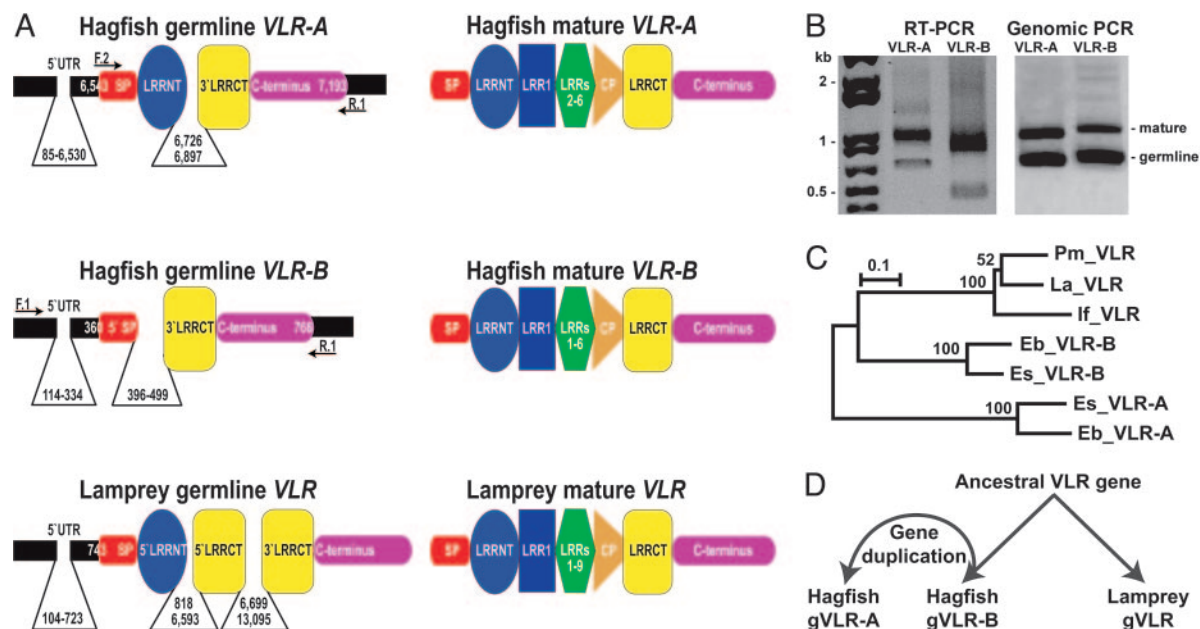
Fig. 4. Agnathan *VLR* genes, transcripts and phylogeny. (*A*) Schematic presentation of germ-line and mature *VLR* genes of Pacific hagfish and Sea lamprey. Colored bars indicate coding regions; size in nucleotides; positions of PCR primers (Table 2) used to amplify hagfish VLR are indicated by arrows and labeled F (forward) R (reverse). (*B*) Pacific hagfish VLRs PCR amplified from lymphocyte-like transcripts (RT-PCR) or blood genomic DNA. Agarose gel image; molecular weight marker indicated on the left (kilobases); position of germ-line and mature VLR amplicons indicated on the right. (*C*) Phylogenetic analysis of agnathan VLRs. Neighbor-joining tree of hagfish and lamprey VLR proteins (same sequences as in Fig. 1); bootstrap values are indicated. Bar represents 10% amino acid divergence. (*D*) Hypothetical model for the evolution of agnathan VLR.

in the genome to provide missing components of the mature *VLR* genes identified in samples of genomic PCR amplicons from lymphocyte-like cells, 35 unique mature *VLR-A* and 38 *VLR-B* sequences from two animals (Fig. 2). Thus the hagfish mature *VLR* genes must be assembled through somatic recombination, as is the case for lamprey, but the mechanism for the recombination process is still unknown.

Germ-line *VLR* genes in hagfish lymphocyte-like cells are actively transcribed before gene rearrangement. PCR amplicons of VLR-A germ-line transcripts are ≈0.7 kb long and ≈0.5 kb long for VLR-B (Fig. 4*B*, RT-PCR; the position of PCR primers is indicated in Fig. 4*A*), whereas the larger amplicons correspond to transcripts from the rearranged mature *VLR* genes, ≈1.1 and ≈0.8 kb for VLR-A and -B, respectively. The corresponding PCR amplicons from blood genomic DNA are ≈0.7 kb for the germ-line genes and ≈1.1 kb for the mature *VLR-A* and *-B* genes (Fig. 4*B*, genomic PCR). In transcripts from germ-line and mature *VLR* genes, the 5′ intron is spliced out to yield RT-PCR products shorter than the corresponding genomic PCR amplicons (see VLR-B in Fig. 4*B*; *gVLR-A* amplicons do not include the 6.4-kb intron). However, the intervening sequences between the coding exons are retained in the germ-line transcripts, because they lack consensus eukaryotic splice sites. The germ-line transcription may be required for *gVLR* rearrangement, as is the case in mammalian antibody class switch recombination, for which germ-line switch region transcription is obligatory (22, 23).

VLR Phylogeny. A phylogenetic analysis of the agnathan VLR proteins reveals three distinct clusters, respectively, composed by lamprey VLR and hagfish VLR-A and -B sequences (Fig. 4*C*). The hagfish VLR-B and lamprey VLR cluster in a separate branch from that with the hagfish VLR-A. The same tree topology was seen when only the VLR diversity regions, LRRNT to LRRCT or LRR1 to CP, were aligned. Hence, either the hagfish *VLR-A* arose by duplication of the ancestral gene (Fig.

4*D*), or the lamprey lost their *VLR-A* ortholog after the split between the hagfish and lamprey lineages, dating 499 ± 38 million years ago in the Cambrian period (24). It is also possible that a lamprey *VLR-A* ortholog exists but was not detected in >18,000 cDNA sequences derived from lamprey lymphocyte-like cells (17), because it is expressed at very low levels or in nonlymphoid cells.

Conclusion

The presence of VLRs in both of the extant cyclostome orders is indicative of strong evolutionary pressure for vertebrates to develop an anticipatory molecular recognition system. Our analysis indicates that, within <40 million years in the Cambrian, two radically different systems evolved in agnathans and gnathostomes in which either LRR or Ig gene fragments undergo recombinatorial assembly to generate diverse repertoires of lymphocyte receptors. This evolutionary scenario raises many intriguing questions, one of which concerns the issue of whether the two adaptive immune strategies represent convergent evolution or whether one was ancestral to the other. Whether VLRs were forerunner vertebrate immune receptors or the rearranging *VLRs* and *Igs* evolved independently will become certain only with an unambiguous resolution of the phylogenetic relationships among the groups of living and extinct jawless and jawed vertebrates (25, 26). In this regard, however, the presence of VLRs in both orders of contemporary agnathans lends additional molecular evidence favoring a monophyletic origin of cyclostomes.

1. Delarbre, C., Gallut, C, Barriel, V., Janvier, P. & Gachelin, G. (2002) *Mol. Phylogenet. Evol.* **22,** 184–192.
2. Furlong, R. F. & Holland, P. W. (2002) *Zool. Sci.* **19,** 593–599.
3. Takezaki, N., Figueroa, F., Zaleska-Rutczynska. Z. & Klein, J. (2003) *Mol. Biol. Evol.* **20,** 287–292.
4. Acton, R. T., Weinheimer, P. F., Hildemann, W. H. & Evans, E. E. (1971) *Infect. Immunol.* **4,** 160–166.
5. Zapata, A., Fange, R., Mattisson, A. & Villena, A. (1984) *Cell Tissue Res.* **235,** 691–693.
6. Gilbertson, P., Wotherspoon, J. & Raison, R. L. (1986) *Dev. Comp. Immunol.* **10,** 1–10.
7. Raison, R. L., Gilbertson, P. & Wotherspoon, J. (1987) *Immunol. Cell Biol.* **65,** 183–188.
8. Newton, R. A., Raftos, D. A., Raison, R. L. & Geczy, C. L. (1994) *Dev. Comp. Immunol.* **18,** 295–303.
9. Raison, R. L., Coverley, J., Hook, J. W., Towns, P., Weston, K. M. & Raftos, D. A. (1994) *Immunol. Cell Biol.* **72,** 326–332.
10. Hildemann, W. H. (1970) *Transplant. Proc.* **2,** 253–259.
11. Pollara, B., Litman, G. W., Finstad, J., Howell, J. & Good, R. A. (1970) *J. Immunol.* **105,** 738–745.
12. Raison, R. L. & Hildemann, W. H. (1984) *Dev. Comp. Immunol.* **8,** 99–108.
13. Good, R. A., Finstad, J. & Litman, G. W. (1972) in *The Biology of Lampreys*, eds Hardisty, M. V. & Potter, I. C. (Academic, London), Vol. II, pp. 405–432.
14. Flajnik, M. F. & Kasahara, M. (2001) *Immunity* **15,** 351–362.
15. Flajnik, M. F. (2002) *Nat. Rev. Immunol.* **2,** 688–698.
16. Suzuki, T., Shin-I, T., Kohara, Y. & Kasahara, M. (2004) *Dev. Comp. Immunol.* **28,** 993–1003.
17. Pancer, Z., Mayer, W. E., Klein, J. & Cooper, M. D. (2004) *Proc. Natl. Acad. Sci. USA* **101,** 13273–13278.
18. Eason, D. D., Cannon, J. P., Haire, R. N., Rast, J. P., Ostrov, D. A. & Litman, G. W. (2004) *Semin. Immunol.* **16,** 215–226.
19. Pancer, Z., Amemiya, C. T., Ehrhardt, G. R. A., Ceitlin, J., Gartland, G. L. & Cooper, M. D. (2004) *Nature* **430,** 174–180.
20. Suzuki, T., Ota, T., Fujiyama, A. & Kasahara, M. (2004) *Genes Genet. Syst.* **79,** 251–253.
21. Kumar, S., Tamura, K. & Nei, M. (2004) *Brief Bioinform.* **5,** 150–163.
22. Bottaro, A., Lansford, R., Xu, L., Zhang, J., Rothman, P. & Alt, F. W. (1994) *EMBO J.* **13,** 665–674.
23. Hein, K., Lorenz, M. G., Siebenkotten, G., Petry, K., Christine, R. & Radbruch, A. (1998) *J. Exp. Med.* **188,** 2369–2374.
24. Hedges, S. B. (2001) in *Major Events in Early Vertebrate Evolution, Systematics Association Special: Molecular Evidence for the Early History of Living Vertebrates*, ed. Ahlberg, P. E. (Taylor & Francis, London), Vol. 61, pp. 119–134.
25. Mallatt, J. & Chen, J. Y. (2003) *J. Morphol.* **258,** 1–31.
26. Meyer, A. & Zardoya, R. (2003) *Annu. Rev. Ecol. Evol. Syst.* **34,** 311–338.

EVOLUTION